Oecologia (2014) 174:631–638
DOI 10.1007/s00442-013-2803-8

PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

The multivariate egg: quantifying within- and among-clutch correlations between maternally derived yolk immunoglobulins and yolk androgens using multivariate mixed models

Erik Postma · Heli Siitari · Hubert Schwabl · Heinz Richner · Barbara Tschirren

Received: 12 March 2013 / Accepted: 3 October 2013 / Published online: 15 October 2013

Abstract Egg components are important mediators of prenatal maternal effects in birds and other oviparous species. Because different egg components can have opposite effects on offspring phenotype, selection is expected to favour their mutual adjustment, resulting in a significant covariation between egg components within and/or among clutches. Here we tested for such correlations between maternally derived yolk immunoglobulins and yolk androgens in great tit (*Parus major*) eggs using a multivariate mixed-model approach. We found no association between yolk immunoglobulins and yolk androgens within clutches, indicating that within clutches the two egg components are deposited independently. Across clutches, however, there was a significant negative relationship between yolk immunoglobulins and yolk androgens, suggesting that selection has co-adjusted their deposition. Furthermore, an experimental manipulation of ectoparasite load affected patterns of covariance among egg components. Yolk immunoglobulins are known to play an important role in nestling immune defence shortly after hatching, whereas yolk androgens, although having growth-enhancing effects under many environmental conditions, can be immunosuppressive. We therefore speculate that variation in the risk of parasitism may play an important role in shaping optimal egg composition and may lead to the observed pattern of yolk immunoglobulin and yolk androgen deposition across clutches. More generally, our case study exemplifies how multivariate mixed-model methodology presents a flexible tool to not only quantify, but also test patterns of (co)variation across different organisational levels and environments, allowing for powerful hypothesis testing in ecophysiology.

Keywords Maternal antibodies · Yolk testosterone · Egg composition · Parasites · Maternal effects

Communicated by Oliver P Love.

E. Postma · B. Tschirren (✉)
Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
e-mail: barbara.tschirren@ieu.uzh.ch

H. Siitari
Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland

H. Schwabl
Centre for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA

H. Richner
Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, 3012 Bern, Switzerland

Introduction

The environment a mother provides for her young early in their life—even before they are born—can have significant and long-lasting consequences for offspring phenotype and performance (Mousseau and Fox 1998; Christians 2002; Monaghan 2008). Such transgenerational phenotypic alterations in response to environmental conditions have been suggested to play a key role in the adaptation to variable, but predictable, environments, and to potentially accelerate evolutionary responses to environmental change (Kirkpatrick and Lande 1989; Cheverud and Moore 1994; Wolf et al. 1998; Räsänen and Kruuk 2007).

Parasites are an environmental factor that can strongly impair host fitness, which requires alterations in host morphology, physiology and/or behaviour to mitigate parasite

impact. Maternal effects have been shown to play an important role in the adaptive alteration of offspring phenotype in response to parasitism (e.g. Heeb et al. 1998; Curno et al. 2009; Ewen et al. 2009). In particular maternally transferred antibodies are crucial for parasite defence early in life. Shortly after birth, the immune system of vertebrates is not yet fully functional and juveniles depend, at least partly, on antibodies received from their mother through placenta, milk or yolk to fight parasites and pathogens (reviewed in Grindstaff et al. 2003; Boulinier and Staszewski 2008; Hasselquist and Nilsson 2009). There are now a number of studies showing that female birds increase yolk antibody concentrations in response to parasitism or experimental immune challenges (Gasparini et al. 2001; Grindstaff 2008), and that maternal antibodies improve specific immune responses and/or provide partial disease protection in the young (Gasparini et al. 2006; Grindstaff et al. 2006; King et al. 2011; Jacquin et al. 2012; Staszewski et al. 2012).

Maternal antibodies are, however, not the only resources mothers can provide to alter offspring fitness. Maternal yolk hormones (in particular androgens), deposited by female birds during egg formation, have been shown to increase growth rate, begging vigour, competitive abilities, aggressiveness and ornamentation (reviewed in Groothuis et al. 2005b; Gil 2008). These traits will, under many social and environmental circumstances, increase offspring survival and/or reproduction. At the same time, however, exposure to high concentrations of maternal yolk androgens before birth may come at a cost in the form of reduced offspring immunocompetence (Hirota et al. 1976; Groothuis et al. 2005a; Müller et al. 2005; Navara et al. 2005, but see e.g. Tschirren et al. 2005; Kankova et al. 2012). Depositing low concentrations of yolk androgens into the eggs when parasite prevalence is expected to be high may therefore increase the offspring's immunological defence capacity. Thereby, immunological costs of prenatal exposure to high yolk androgen concentrations might contribute to the maintenance of variation in yolk hormone deposition in natural populations experiencing variation in parasite prevalence (Tschirren et al. 2009a). Indeed, parasites have been identified as an environmental factor that influences yolk hormone deposition in the wild (Tschirren et al. 2004; Gil et al. 2006).

Hen fleas (*Ceratophyllus gallinae*) are common, nest-based ectoparasites of hole-breeding passerines (Tripet and Richner 1997). Because fleas overwinter in the old nesting material, and are therefore present before the female starts egg laying (Tripet et al. 2002), females can, at least in theory, predict the future flea load of their offspring. This makes the bird-flea host-parasite system an ideal model to test for adaptive maternal effects in response to parasitism. In line with the hypothesis that mothers adaptively alter egg composition in response to parasitism, previous studies have shown that an experimental manipulation of flea load in the nests of great tits (*Parus major*) before egg laying lead to the deposition of higher concentrations of maternal yolk immunoglobulins (Buechler et al. 2002) and lower concentrations of maternal yolk androgens (Tschirren et al. 2004). Based on these findings, we hypothesise that females should either produce eggs with high immunoglobulin and low androgen concentrations *or* eggs with low immunoglobulin and high androgen concentrations, and that the optimal strategy depends on parasite prevalence. We thus predict that selection has co-adjusted the deposition of these two egg components to increase offspring and/or maternal fitness, resulting in a negative correlation between immuno-enhancing immunoglobulins and immunosuppressive androgens (see also Groothuis et al. 2006; Hargitai et al. 2009). Because nestlings within a nest are likely to be exposed to similar levels of parasitism, whereas parasite load can vary substantially among nests, we expect this negative correlation to be particularly pronounced at the among-clutch level.

Here we tested this prediction by quantifying among- and within-clutch correlations of yolk immunoglobulin and yolk androgen deposition in great tit eggs using a multivariate mixed-model approach. This approach provides a powerful and flexible statistical framework to both quantify and test patterns of variation and covariation across different organisational levels and environments.

Materials and methods

Study population and parasite manipulation

The study was performed in a great tit population breeding in nest boxes in a deciduous forest in Switzerland. During winter, all old nesting material was removed from the boxes to ensure that boxes were parasite free. When the new breeding season started, we monitored nest-building activity and manipulated the load of nest-based ectoparasites (hen fleas, *Ceratophyllus gallinae*) once the nest cup was constructed (7.7 ± 0.6 days before the female laid the first egg). All nests were then heat treated in a microwave oven following Richner et al. (1993) to kill fleas that immigrated into the boxes during nest building. Afterwards, nests were randomly assigned to be either infested with 25 female and 15 male hen fleas or to remain free of parasites. Hen fleas used for the manipulation were extracted from old nests collected in the study area during winter. After the manipulation, we visited the boxes daily to determine the start of egg laying. The first, third, sixth and then every other egg, and the last egg of a clutch was collected on the day it was laid. Collected eggs were immediately replaced with

an artificial egg. In total, we collected 300 eggs from 27 parasitized and 27 parasite-free nests. Yolks were separated from the albumen and frozen at −20 °C until hormone and immunoglobulin (IgG) analysis. The experiments were conducted under a license provided by the Ethical Committee of the Office of Agriculture, Bern, Switzerland.

Yolk androgen assays

Yolks were homogenized with an equal (1 µl per mg of yolk) amount of distilled water. Aliquots of this yolk–water emulsion (250 mg) were used for the quantification of yolk androstenedione (A4), yolk testosterone (T) and yolk 5α-dihydrotestosterone (DHT) concentrations using radioimmunoassay (RIA). The extraction of androgens and RIA procedures were performed according to published protocols (see Schwabl 1993; Tschirren et al. 2004 for details).

Yolk IgG assays

Yolk IgG concentrations were determined using enzyme-linked immunosorbent assay (ELISA) as described in Tschirren et al. (2009b). In brief, we centrifuged the homogenised yolk samples and collected the clear supernatant containing the immunoglobulins for analysis. Ninety-six-well ELISA plates (Nunc Immunoplate) were coated with anti-chicken IgG (Sigma C-6409) 1:180 in 50 mM carbonate buffer (pH 9.6). The plates were then incubated at 4 °C overnight. After emptying the wells, they were masked with 1 % bovine serum albumin–phosphate buffered saline (BSA-PBS; Roche Diagnostics) for 1 h and washed three times with 200 µl with PBS-Tween. Samples and their replicates (50 µl/well) were diluted with 1 % BSA-PBS and added to the wells (dilutions of 1:2,000 and 1:4,000 of the supernatant).

To determine total IgG levels, a standard of pooled yolks (50 µl/well; diluted with 1 % BSA-PBS) was added to each plate, and was assigned a concentration of 10^6. All values were subsequently expressed relative to this standard. Samples and standards were incubated for 3 h at room temperature. After washing the plates (three times with 200 µl PBS-Tween), an alkaline phosphatase conjugated antibody (Sigma A-971 anti-chicken IgG; diluted with 1 % BSA-PBS 1:10,000) was added to the wells and they were incubated overnight at 4 °C. Finally, after washing the wells (three times with 400 µl PBS-Tween) an alkaline phosphatase substrate, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) in 1 M diethanol amine buffer (1 mg/ml) was applied (50 µl/well). The absorbance of the wells was read in an ELISA reader at 405 nm for up to 1 h (or until the highest standard reached the absorbance 2.0).

Statistical analyses

Ecophysiology frequently deals with inherently multivariate data, yet multivariate mixed-model approaches are still relatively rarely used to test for differences in the associations among multiple physiological traits across different organisational levels and environments. Here we used a multivariate mixed-model approach to test whether correlations between maternally derived yolk IgG and yolk androgens are significantly different from zero, whether the within- and among-clutch correlations are significantly different from each other, and whether they are affected by the presence of parasites. To this end, we simultaneously estimated all within- and among-nest (co)variances and correlations by fitting a restricted maximum likelihood multivariate mixed model, with yolk IgG and the three yolk androgens A4, T and DHT as the dependent variables, and female identity (nested in parasite treatment) as a random effect.

Yolk IgG (U/mg yolk) and androgen concentrations (pg/mg yolk) were log transformed and standardized for the statistical analyses. The significance of each within- and among-clutch correlation was tested by constraining it to zero, and testing whether this resulted in a significantly worse fit of the model to the data using a likelihood-ratio test with 1 *df* (Pinheiro and Bates 2000). To avoid the statistical complications associated with multiple testing and to maximise statistical power, we furthermore compared a model, in which all within- or among-clutch correlations involving yolk IgG and the three yolk androgens were constrained to zero, to an unconstrained model using a likelihood-ratio test with 3 *df*.

Using a similar approach, we tested whether the within- and among-clutch correlations were significantly different from each other by comparing a model, in which within- and among-clutch correlations were constrained to be the same, to an unconstrained model. Again, we did this both for all three correlations involving yolk IgG and yolk androgens at once (likelihood-ratio test with 3 *df*) and for all combinations of traits separately (likelihood-ratio test with 1 *df*).

In a last step, we tested whether the relationship between yolk IgG and yolk androgens was affected by the parasite treatment, providing insights into the environmental (i.e. parasite) dependency of within- and among-clutch patterns of (co)variance. To this end, (log-transformed and standardised) yolk IgG and yolk androgen concentrations from nests with and without parasites were considered separate traits, resulting in a total of eight dependent variables. Because each clutch was either from a parasitized or from a parasite-free nest, the correlations among yolk IgG and yolk androgen concentrations across treatments cannot be estimated and had to be constrained to zero. This results

in a total of eight variances and 12 correlations to be estimated. Although this provides us with (co)variance and correlation estimates that are identical to those from two separate treatment-specific models, our approach makes it possible to directly test whether the correlations between yolk IgG and yolk androgens are significantly different in the absence or presence of parasites (i.e. similar to testing for an interaction with parasite treatment). As in the overall analyses outlined above, this is achieved by constraining one or more correlations with and without parasites to be the same, and comparing its loglikelihood value to that of an unconstrained model.

Although all significance testing is based on likelihood-ratio tests, approximate SEs for all variance ratios and correlations were estimated for completeness. All mixed-model analyses were performed in ASReml 3 (Gilmour et al. 2009). Means ± 1 SE are presented.

Results

Correlations between yolk IgG and yolk hormones within and among clutches

The complete correlation matrix (including approximate SEs) is given in Table 1. In short, clutch (i.e. female) identity explained a large proportion of variation in maternal yolk IgG (70.1 ± 4.8 %), yolk A4 (62.0 ± 5.7 %), yolk T (63.8 ± 5.5 %) and yolk DHT (66.1 ± 5.3 %) concentrations. There was a consistent positive relationship between A4 and T, whereas the relationship between DHT and the other androgens was weaker and more variable.

Among clutches, there was a significant negative association between maternal yolk IgG and yolk androgen concentrations ($\chi^2 = 10.38$, $df = 3$, $P = 0.016$). Androgen-specific estimates revealed that this negative association was to a large degree driven by a negative correlation between yolk IgG and yolk DHT ($\chi^2 = 4.67$, $df = 1$, $P = 0.031$). However, although the associations with yolk T ($\chi^2 = 0.30$, $df = 1$, $P = 0.585$) and yolk A4 ($\chi^2 = 1.87$, $df = 1$, $P = 0.171$) were weaker and by themselves not significantly different from zero, they were negative as well (Fig. 1).

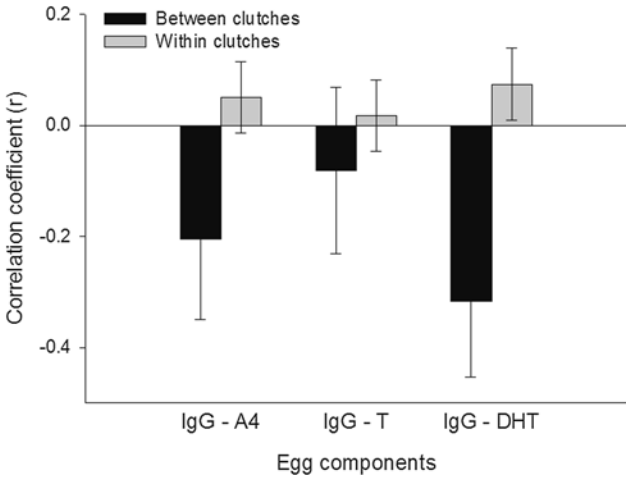

Fig. 1 Correlation coefficients (r ± 1 SE) of the relationship between maternal yolk immunoglobulin (*IgG*) and yolk androstenedione (*A4*), yolk testosterone (*T*) and yolk 5α-dihydrotestosterone (*DHT*) concentrations among (*black bars*) and within (*grey bars*) great tit clutches

Within clutches, maternal yolk IgG and maternal yolk androgen concentrations were not significantly correlated ($\chi^2 = 2.64$, $df = 3$, $P = 0.450$). Androgen-specific estimates revealed weak positive, but non-significant, correlations between maternal yolk IgG and yolk A4 ($\chi^2 = 0.61$, $df = 1$, $P = 0.435$), yolk T ($\chi^2 = 0.07$, $df = 1$, $P = 0.794$) and yolk DHT ($\chi^2 = 1.25$, $df = 1$, $P = 0.264$) concentrations (Fig. 1; Table 1). When comparing the within- and among-clutch correlations between maternal yolk IgG and yolk androgen concentrations, we found that they were significantly different from each another ($\chi^2 = 12.67$, $df = 3$, $P = 0.005$; Fig. 1).

Table 1 The association between (log-transformed and standardised) yolk immunoglobulin (*IgG*) and yolk androgen concentrations

	A4	T	DHT	IgG
A4	62.0 (5.7)	0.747 (0.028)*	−0.075 (0.064)	0.050 (0.064)[a]
T	0.808 (0.054)*	63.8 (5.5)	0.100 (0.064)	0.017 (0.064)[a]
DHT	0.007 (0.153)	0.227 (0.145)	66.1 (5.3)*	0.074 (0.065)[a]
IgG	−0.204 (0.145)[a]	−0.082 (0.153)[a]	−0.317 (0.137)[a]	70.1 (4.8)

The percentage of variance among clutches is given on the *diagonal*. Among-clutch correlations are provided *below the diagonal*, and within-clutch correlations are provided *above the diagonal*. Approximate SEs are given *in parentheses*

A4 Androstenedione, *T* testosterone, *DHT* 5α-dihydrotestosterone

* Correlations are significantly different from zero at the 5 % level (based on likelihood-ratio tests)

[a] Correlations involving IgG

Effects of parasite treatment on the correlations between yolk IgG and yolk androgen concentrations

The complete treatment-specific correlation matrices are given in Table 2. Among clutches, we found that the correlations between maternal yolk IgG and yolk androgens tended to be different between flea-free and flea-infested nests ($\chi^2 = 6.93$, $df = 3$, $P = 0.074$). Androgen-specific estimates revealed that this difference was mainly due to a significantly negative correlation between maternal yolk IgG and yolk A4 in flea-free nests ($\chi^2 = 6.91$, $df = 1$, $P = 0.009$), but a weak and non-significant positive correlation in flea-infested nests ($\chi^2 = 0.21$, $df = 1$,

Table 2 The association between (log-transformed and standardised) yolk IgG and yolk androgen concentrations in the absence and presence of fleas

	A4	T	DHT	IgG
Fleas absent				
A4	61.8 (8.2)	0.842 (0.027)*	−0.007 (0.093)	0.017 (0.093)[a]
T	0.753 (0.095)*	58.6 (8.6)	0.185 (0.090)*	0.069 (0.092)[a]
DHT	−0.210 (0.220)	0.042 (0.230)	51.9 (9.1)	0.238 (0.088)[a*]
IgG	−0.521 (0.159)[a*]	−0.279 (0.199)[a]	−0.164 (0.213)[a]	82.1 (4.7)
Fleas present				
A4	60.2 (8.2)	0.665 (0.050)*	−0.144 (0.087)	0.079 (0.088)[a]
T	0.847 (0.066)*	66.7 (7.4)	0.019 (0.089)	−0.010 (0.089)[a]
DHT	0.225 (0.206)	0.388 (0.182)	75.6 (6.0)	−0.070 (0.091)[a]
IgG	0.103 (0.224)[a]	0.004 (0.224)[a]	−0.474 (0.177)[a*]	48.5 (8.8)

For both absence and presence of fleas, the percentage of variance among clutches is given on the *diagonal*, correlations among clutches are provided *below the diagonal* and correlations within clutches *above the diagonal*. Approximate SEs are provided *in parentheses*. For abbreviations, see Table 1

* Correlations are significantly different from zero at the 5 % level (based on likelihood-ratio tests)

[a] Correlations involving yolk IgG concentration

$P = 0.644$; difference in the correlation between yolk IgG and yolk A4 between treatment groups, $\chi^2 = 4.72$, $df = 1$, $P = 0.030$). Although the correlation between IgG and yolk T was stronger in flea-free nests than in flea-invested nests, they were not significantly different from each other ($\chi^2 = 0.88$, $df = 1$, $P = 0.348$). Finally, the correlation between maternal yolk IgG and yolk DHT was negative in both flea-infested and flea-free nests, and although the correlation was stronger in flea-invested nests, the two correlations were again not significantly different from each other ($\chi^2 = 1.23$, $df = 1$, $P = 0.268$).

Within clutches, the correlation between maternal yolk IgG and yolk androgens tended to be more pronounced in flea-free than in flea-infested nests ($\chi^2 = 6.54$, $df = 3$, $P = 0.088$). Androgen-specific estimates showed that this difference was mainly due to the correlation between maternal yolk IgG and yolk DHT, which was significantly positive in flea-free nests ($\chi^2 = 6.63$, $df = 1$, $P = 0.010$), but weakly and non-significantly negative in flea-infested nests ($\chi^2 = 0.56$, $df = 1$, $P = 0.453$; difference in the correlation between yolk IgG and yolk DHT between treatment groups, $\chi^2 = 5.55$, $df = 1$, $P = 0.019$). For maternal yolk IgG and yolk A4 ($\chi^2 = 0.23$, $df = 1$, $P = 0.633$) and maternal yolk IgG and yolk T ($\chi^2 = 0.38$, $df = 1$, $P = 0.540$), the correlations were weak and did not differ significantly between treatment groups.

Discussion

We have shown that maternal yolk immunoglobulin and yolk androgen concentrations [and in particular yolk DHT, which has the highest affinity for the androgen receptor and hence the greatest potency of all three androgens (Groothuis and Schwabl 2008)] correlate negatively across great tit clutches, which is in line with selection having favoured the mutual adjustment of these egg components. Indeed, maternally derived immunoglobulins play an important role in immune defence against pathogens early in a bird's life (reviewed in Grindstaff et al. 2003; Boulinier and Staszewski 2008; Hasselquist and Nilsson 2009; but see Tschirren et al. 2009b), whereas maternally derived yolk androgens can be immunosuppressive (Groothuis et al. 2005a; Müller et al. 2005; Navara et al. 2005, but see e.g. Tschirren et al. 2005; Kankova et al. 2012). Parasite-mediated selection might therefore play an important role in shaping optimal egg composition, and lead to a negative correlation between these egg components. Alternatively to the scenario outlined above, the co-adjustment of immunoglobulins and androgens might be beneficial for the mother, rather than the offspring, and the patterns observed in the eggs might simply reflect a passive, and not necessarily adaptive transfer from the mother's circulation (but see Heylen et al. 2012). Although the question whether the concentrations of maternally derived immunoglobulins and androgens in the eggs are a reflection of the concentrations present in the mother's circulation is still debated, there is evidence that the deposition of egg components is, at least to a certain degree, independent of the female's own circulation (reviewed in Groothuis and Schwabl 2008; Hasselquist and Nilsson 2009).

Interestingly, a significantly different pattern between yolk immunoglobulins and yolk androgens was observed *within* clutches. For both yolk immunoglobulin as well as

yolk androgen concentrations, most variation was found among clutches, whereas within-clutch variation was comparably low. This low variation reduces the potential for within-clutch covariation between egg components. However, it is important to note that it does not explain the lack of a correlation, which is independent of the within-clutch variances. Instead, the lack of a (negative) correlation within clutches may at least partly be explained by selection for different patterns of deposition at the within-clutch level. Indeed, whereas there is no change in IgG concentrations with laying order ($F_{1,248.3} = 0.131$, $P = 0.718$), yolk A4 and T significantly increase with laying order, whereas yolk DHT concentrations significantly decrease (Tschirren et al. 2004). Differences in yolk hormone deposition with laying order have been suggested to promote brood reduction (in the case of decreasing hormone concentrations) or increase competitive abilities of late-hatched nestlings (in the case of increasing hormone concentrations with laying order) (Schwabl 1993; Schwabl et al. 1997). In line with the latter hypothesis we found in a previous study that it was particularly nestlings that hatched late in the laying sequence that benefited from high yolk androgen concentrations (Tschirren et al. 2005). Unlike yolk immunoglobulins, yolk androgens may thus be strategically deposited within a clutch to optimise growth trajectories. Importantly, the lack of a correlation between yolk immunoglobulins and androgens *within* clutches shows that mothers can, at least within certain limits, vary each of the egg components independently, or that they are transferred randomly. It thereby provides indirect support for the hypothesis that selection has favoured the mutual adjustment of yolk immunoglobulins and yolk androgens *among* clutches.

The mechanisms underlying the observed negative correlation among clutches are currently unknown. One possibility is a negative genetic correlation between yolk immunoglobulin and yolk androgen deposition (Schroderus et al. 2010). Extensive pedigree data (Postma and Charmantier 2007; Wilson et al. 2010), or the creation of selection lines for high/low yolk immunoglobulin deposition, and testing for correlated responses in yolk androgens (or vice versa) (Schroderus et al. 2010), would make it possible to estimate the degree to which the negative correlation between the two egg components has a genetic basis. Furthermore, environmental factors that simultaneously, but oppositely, affect immunoglobulin and androgen deposition (e.g. parasite exposure or health status of the mother (Boonekamp et al. 2008)) might also play a role. Further experimental work is required to quantify the role of such factors in mediating the negative covariation of yolk androgens and antibodies observed across clutches. Possible approaches include the experimental manipulation of female health status or condition, and to test how this affects the correlation between immunoglobulins and androgens in the eggs (see e.g. Gasparini et al. 2007). As a further improvement to our current study, females could be injected with a novel antigen (e.g. tetanus vaccine) to measure specific antibody responses, rather than total IgG.

In contrast to our study, previous work did not detect a significant association between yolk immunoglobulin and yolk androgen concentrations in the eggs of free-living birds (Groothuis et al. 2006; Hargitai et al. 2009) or observed positive relationship between IgG and A4 within clutches (Gasparini et al. 2007). These differences may be explained by ecological differences among species (e.g. differences in parasite-mediated selection pressures), or a focus on within-clutch variation in previous work.

Covariation among other egg components, however, is well documented. Total yolk carotenoid concentration, for example, is positively correlated with vitamin E concentration within collared flycatcher (*Ficedula albicollis*) clutches (Hargitai et al. 2006). Similar positive relationships were found between carotenoid, vitamin E and vitamin A concentrations within, and, to a lesser extent, among clutches of yellow-legged gulls (*Larus michahellis*) (Rubolini et al. 2011). Finally, there was a negative within-clutch correlation between yolk antioxidants and yolk androgens in black-backed gulls (*Larus fuscus*) (Royle et al. 2001).

In addition to the overall within- and among-clutch patterns of covariation between yolk immunoglobulins and yolk androgens, we have here also tested how an experimental parasite-infestation influences the strength and direction of this relationship, again both at the within- and among-clutch level. This provides us with first insights into the environmental dependency of these correlations. Indeed, we found some indication for correlations among egg components to depend on the presence or absence of parasites (e.g. the relationship between yolk IgG and yolk DHT becomes more negative in parasite-infested nests), which is deserving of future study. In particular, it would be interesting to separate the among-clutch correlations into their genetic and environmental components (see above), and to test how the genetic correlation between yolk IgG and yolk androgens is shaped by the environment [i.e. to test for gene × environment interactions (Sgrò and Hoffmann 2004)]. The latter is particularly interesting given that, if parasites are an important selective force acting upon these egg components, such a parasite dependency of the genetic variance-covariance matrix could have major evolutionary implications. Having said this, it should be noted that overall the differences between the two treatment groups did not reach statistical significance ($P = 0.074$ and $P = 0.088$ for the among- and within-clutch comparisons, respectively), and in the absence of more data we have to be careful not to over-interpret the observed patterns.

The treatment-specific analysis of within- and among-clutch correlations provides an illustration of the

possibilities offered by the multivariate mixed-model approach taken here. Although its strengths when it comes to the estimation of within- and among-clutch patterns of variance and covariance have been highlighted before (e.g. Rubolini et al. 2011), we would here like to emphasise the advantages offered in terms of hypothesis testing. First, as the number of parameters to be estimated increases exponentially with the number of traits analysed, individual parameters are typically estimated with low precision. Although as a consequence none of the individual parameters may be significantly different from zero, being able to simultaneously constrain multiple elements to zero (in this case, all correlations involving yolk IgG and the different yolk androgens) allowed us to maximise statistical power and avoid problems associated with multiple testing. Second, by constraining particular elements of the (within- or among-clutch) correlation matrix to be identical, it is possible to explicitly test whether covariances and/or correlations differ significantly across organisational levels (here within versus among clutch) or environments (here with or without fleas). Third, it is possible to simultaneously correct all traits for the same or a different set of fixed effects, which is not possible in a univariate regression analysis, in which confounding variation can only be accounted for in the dependent variable, but not in the explanatory covariates.

Although routinely employed in other fields, including quantitative genetics (e.g. Postma et al. 2011), within the light of these advantages it is surprising that multivariate mixed-model methodology is still relatively rarely used in other fields, including that of ecophysiology, which routinely deal with inherently multivariate and correlated phenotypes.

Acknowledgments The study was financially supported by the Swiss National Science Foundation (grant no. 31-53956.98 to H. R. and PP00P3_128386 to B. T.).

References

Rubolini D et al (2011) Maternal effects mediated by egg quality in the yellow-legged gull *Larus michahellis* in relation to laying order and embryo sex. Front Zool 8:24 doi:10.1186/1742-9994-1188-1124

Boonekamp JJ, Ros AHF, Verhulst S (2008) Immune activation suppresses plasma testosterone level: a meta-analysis. Biol Lett 4:741–744

Boulinier T, Staszewski V (2008) Maternal transfer of antibodies: raising immuno-ecology issues. Trends Ecol Evol 23:282–288

Buechler K, Fitze PS, Gottstein B, Jacot A, Richner H (2002) Parasite-induced maternal response in a natural bird population. J Anim Ecol 71:247–252

Cheverud JM, Moore AJ (1994) Quantitative genetics and the role of the environment provided by relatives in behavioral evolution. In: Boake CRB (ed) Quantitative genetic studies of behavioral evolution. The University of Chicago Press, Chicago, pp 67–100

Christians JK (2002) Avian egg size: variation within species and inflexibility within individuals. Biol Rev 77:1–26

Curno O, Behnke JM, McElligott AG, Reader T, Barnard CJ (2009) Mothers produce less aggressive sons with altered immunity when there is a threat of disease during pregnancy. Proc R Soc B 276:1047–1054

Ewen JG, Thorogood R, Brekke P, Cassey P, Karadas F, Armstrong DP (2009) Maternally invested carotenoids compensate costly ectoparasitism in the hihi. Proc Natl Acad Sci USA 106:12798–12802

Gasparini J, McCoy KD, Haussy C, Tveraa T, Boulinier T (2001) Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi* sensu lato in a colonial seabird, the kittiwake *Rissa tridactyla*. Proc R Soc B 268:647–650

Gasparini J, McCoy KD, Staszewski V, Haussy C, Boulinier T (2006) Dynamics of anti-*Borrelia* antibodies in blacklegged kittiwake (*Rissa tridactyla*) chicks suggest a maternal educational effect. Can J Zool 84:623–627

Gasparini J, Boulinier T, Gill VA, Gil D, Hatch SA, Roulin A (2007) Food availability affects the maternal transfer of androgens and antibodies into eggs of a colonial seabird. J Evol Biol 20: 874–880

Gil D (2008) Hormones in avian eggs: physiology, ecology and behavior. In: Brockmann HJ, Roper TJ, Naguib M, Wynne-Edwards KE, Barnard C, Mitani J (eds) Advances in the study of behavior, vol 38. Elsevier, Amsterdam, pp 337–398

Gil D, Marzal A, de Lope F, Puerta M, Moller AP (2006) Female house martins (*Delichon urbica*) reduce egg androgen deposition in response to a challenge of their immune system. Behav Ecol Sociobiol 60:96–100

Gilmour AR, Gogel BJ, Cullis BR, Thompson R (2009) ASReml: user guide Release 3.0. VSN International, Hemel Hempstead, UK

Grindstaff JL (2008) Maternal antibodies reduce costs of an immune response during development. J Exp Biol 211:654–660

Grindstaff JL, Brodie ED III, Ketterson ED (2003) Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. Proc R Soc B 270:2309–2319

Grindstaff JL, Hasselquist D, Nilsson JA, Sandell M, Smith HG, Stjernman M (2006) Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proc R Soc B 273:2551–2557

Groothuis TGG, Schwabl H (2008) Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? Phil Trans R Soc B 363:1647–1661

Groothuis TGG, Eising CM, Dijkstra C, Müller W (2005a) Balancing between costs and benefits of maternal hormone deposition in avian eggs. Biol Lett 1:78–81

Groothuis TGG, Müller W, von Engelhardt N, Carere C, Eising C (2005b) Maternal hormones as a tool to adjust offspring phenotype in avian species. Neurosci Biobehav Rev 29:329–352

Groothuis TGG et al (2006) Multiple pathways of maternal effects in black-headed gull eggs: constraint and adaptive compensatory adjustment. J Evol Biol 19:1304–1313

Hargitai R, Matus Z, Hegyi G, Michl G, Toth G, Török J (2006) Antioxidants in the egg yolk of a wild passerine: differences between breeding seasons. Comp Biochem Physiol B 143:145–152

Hargitai R, Arnold KE, Herényi M, Prechl J, Török J (2009) Egg composition in relation to social environment and maternal physiological condition in the collared flycatcher. Behav Ecol Sociobiol 63:869–882

Hasselquist D, Nilsson JA (2009) Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. Phil Trans R Soc B 364:51–60

Heeb P, Werner I, Kölliker M, Richner H (1998) Benefits of induced host responses against an ectoparasite. Proc R Soc B 265:51–56

Heylen D, Muller W, Groothuis TGG, Matthysen E (2012) Female great tits do not alter their yolk androgen deposition when infested with a low-transmittable ectoparasite. Behav Ecol Sociobiol 66:287–293

Hirota Y, Suzuki T, Chazono Y, Bito Y (1976) Humoral immune responses characteristic of testosterone propionate treated chickens. Immunology 30:341–348

Jacquin L, Blottiere L, Haussy C, Perret S, Gasparini J (2012) Prenatal and postnatal parental effects on immunity and growth in 'lactating' pigeons. Funct Ecol 26:866–875

Kankova Z, Zeman M, Okuliarova M (2012) Growth and innate immunity are not limited by selection for high egg testosterone content in Japanese quail. J Exp Biol 215:617–622

King MO, Owen JP, Schwabl H (2011) Injecting the mite into ecological immunology: measuring the antibody response of house sparrows (*Passer domesticus*) challenged with hematophagous mites. Auk 128:340–345

Kirkpatrick M, Lande R (1989) The evolution of maternal characters. Evolution 43:485–503

Monaghan P (2008) Early growth conditions, phenotypic development and environmental change. Phil Trans R Soc B 363:1635–1645

Mousseau TA, Fox CW (1998) Maternal effects as adaptations. Oxford University Press, New York

Müller W, Groothuis TGG, Kasprzik A, Dijkstra C, Alatalo RV, Siitari H (2005) Prenatal androgen exposure modulates cellular and humoral immune function of black-headed gull chicks. Proc R Soc B 272:1971–1977

Navara KJ, Hill GE, Mendonca MT (2005) Variable effects of yolk androgens on growth, survival, and immunity in eastern bluebird nestlings. Physiol Biochem Zool 78:570–578

Pinheiro JC, Bates DM (2000) Mixed-effects models in S and S-Plus. Springer, New York

Postma E, Charmantier A (2007) What 'animal models' can and cannot tell ornithologists about the genetics of wild populations. J Ornithol 148:S633–S642

Postma E, Spyrou N, Rollins LA, Brooks RC (2011) Sex-dependent selection differentially shapes genetic variation on and off the guppy Y chromosome. Evolution 65:2145–2156

Räsänen K, Kruuk LEB (2007) Maternal effects and evolution at ecological time-scales. Funct Ecol 21:408–421

Richner H, Oppliger A, Christe P (1993) Effect of an ectoparasite on reproduction in great tits. J Anim Ecol 62:703–710

Royle NJ, Surai PF, Hartley IR (2001) Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? Behav Ecol 12:381–385

Schroderus E et al (2010) Intra- and intersexual trade-offs between testosterone and immune system: implications for sexual and sexually antagonistic selection. Am Nat 176:E90–E97

Schwabl H (1993) Yolk is a source of maternal testosterone for developing birds. Proc Natl Acad Sci USA 90:11446–11450

Schwabl H, Mock DW, Gieg JA (1997) A hormonal mechanism for parental favouritism. Nature 386:231

Sgrò CM, Hoffmann AA (2004) Genetic correlations, tradeoffs and environmental variation. Heredity 93:241–248

Staszewski V, Reece SE, O'Donnell AJ, Cunningham EJA (2012) Drug treatment of malaria infections can reduce levels of protection transferred to offspring via maternal immunity. Proc R Soc B 279:2487–2496

Tripet F, Richner H (1997) The coevolutionary potential of a 'generalist' parasite, the hen flea *Ceratophyllus gallinae*. Parasitology 115:419–427

Tripet F, Jacot A, Richner H (2002) Larval competition affects the life histories and dispersal behavior of an avian ectoparasite. Ecology 83:935–945

Tschirren B, Richner H, Schwabl H (2004) Ectoparasite-modulated deposition of maternal androgens in great tit eggs. Proc R Soc B 271:1371–1375

Tschirren B, Saladin V, Fitze PS, Schwabl H, Richner H (2005) Maternal yolk testosterone does not modulate parasite susceptibility or immune function in great tit nestlings. J Anim Ecol 74:675–682

Tschirren B, Sendecka J, Groothuis TGG, Gustafsson L, Doligez B (2009a) Heritable variation in maternal yolk hormone transfer in a wild bird population. Am Nat 174:557–564

Tschirren B, Siitari H, Saladin V, Richner H (2009b) Transgenerational immunity in a bird-ectoparasite system: do maternally transferred antibodies affect parasite fecundity or the offspring's susceptibility to fleas? Ibis 151:160–170

Wilson AJ, et al. (2010) An ecologist's guide to the animal model. J Anim Ecol 79:13–26

Wolf JB, Brodie ED, Cheverud JM, Moore AJ, Wade MJ (1998) Evolutionary consequences of indirect genetic effects. Trends Ecol Evol 13:64–69